

Supplementary material for

A single point mutation blocks the entrance of ligands to the cannabinoid CB₂ receptor via the lipid bilayer

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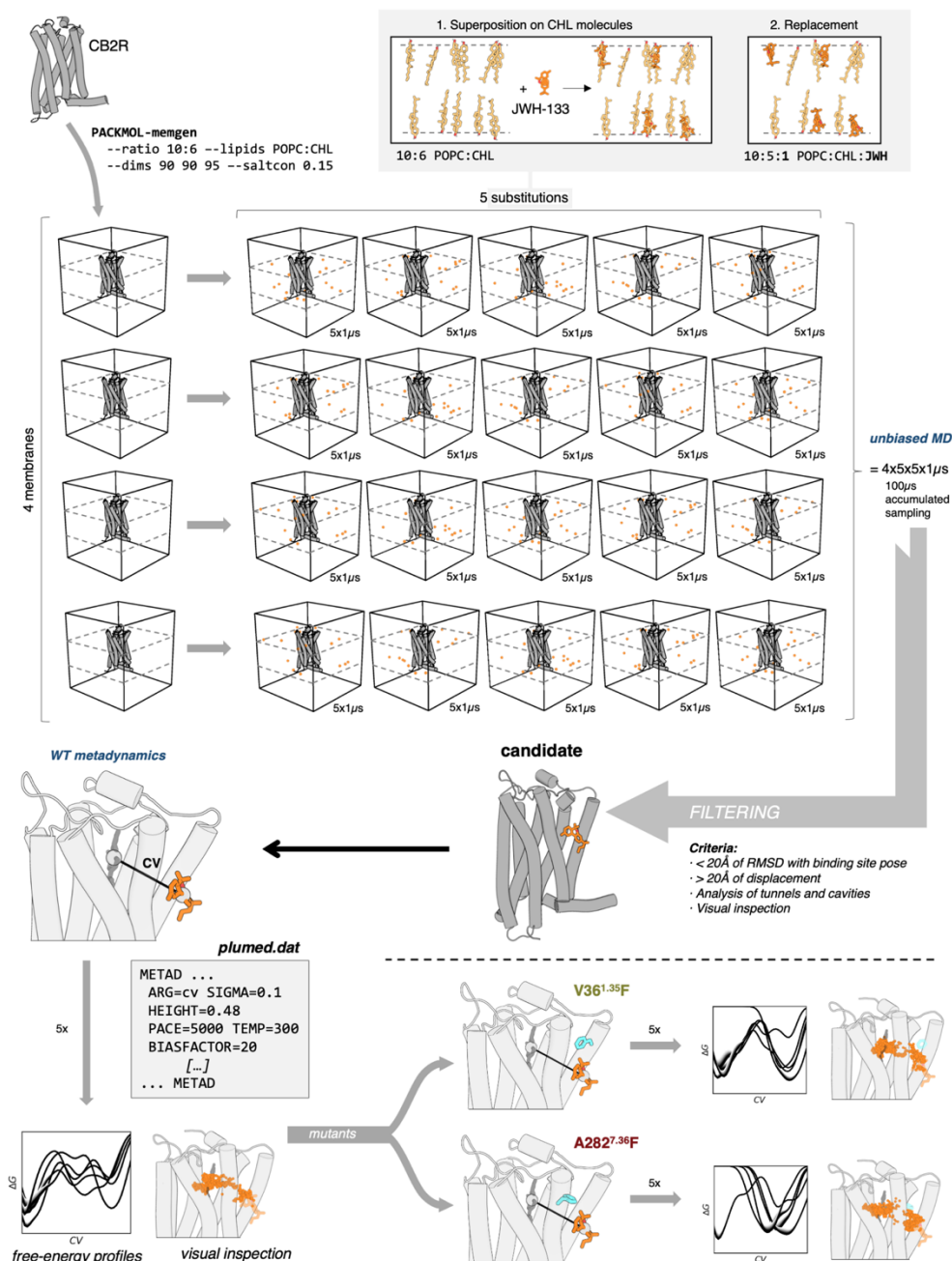


Figure S1. Workflow of the computational process. The inactive structure of CB₂R (PDB id 5ZTY) was given as an input to PACKMOL-memgen to generate four original membrane systems (commands are shown) with a 10:6 POPC:CHL ratio. A random substitution of CHL by JWH-133 (JWH) was performed five times, leading to a 10:5:1 POPC:CHL:JWH ratio. Each of these twenty combinations was subjected to five replicas of unbiased 1μs MD simulation with an aggregate sampling of 100 μs. A filter (rmsd < 20Å relative to the reference docked binding mode + > 20 Å of displacement from the initial position) was applied to the data collected every 10 ns (10,000 snapshots). The structures that spontaneously bound the tunnel between TMs 1 & 7 were selected for WT metadynamics simulations. The CV of choice was the

distance between centers of mass of JWH-133 at the initial (lipid-facing cavity) and final (orthosteric site) points. Five replicas were calculated, using a plumed.dat input file with the depicted settings. Similar simulations were performed for the Val36^{1.35}Phe and Ala282^{7.36}Phe mutants.

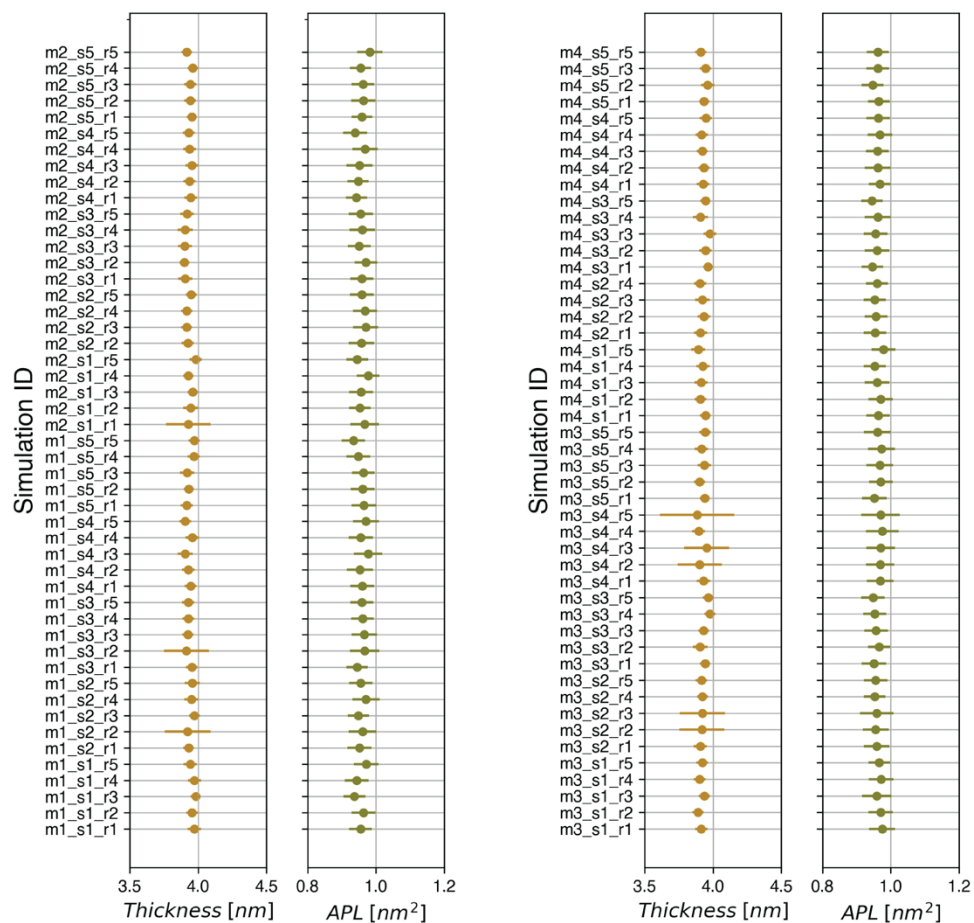


Figure S2. Analysis of membrane stability during the one hundred unbiased 1 μ s MD simulations. The lipid bilayer thickness and the area per lipid (APL), calculated with FATSLiM, are shown.

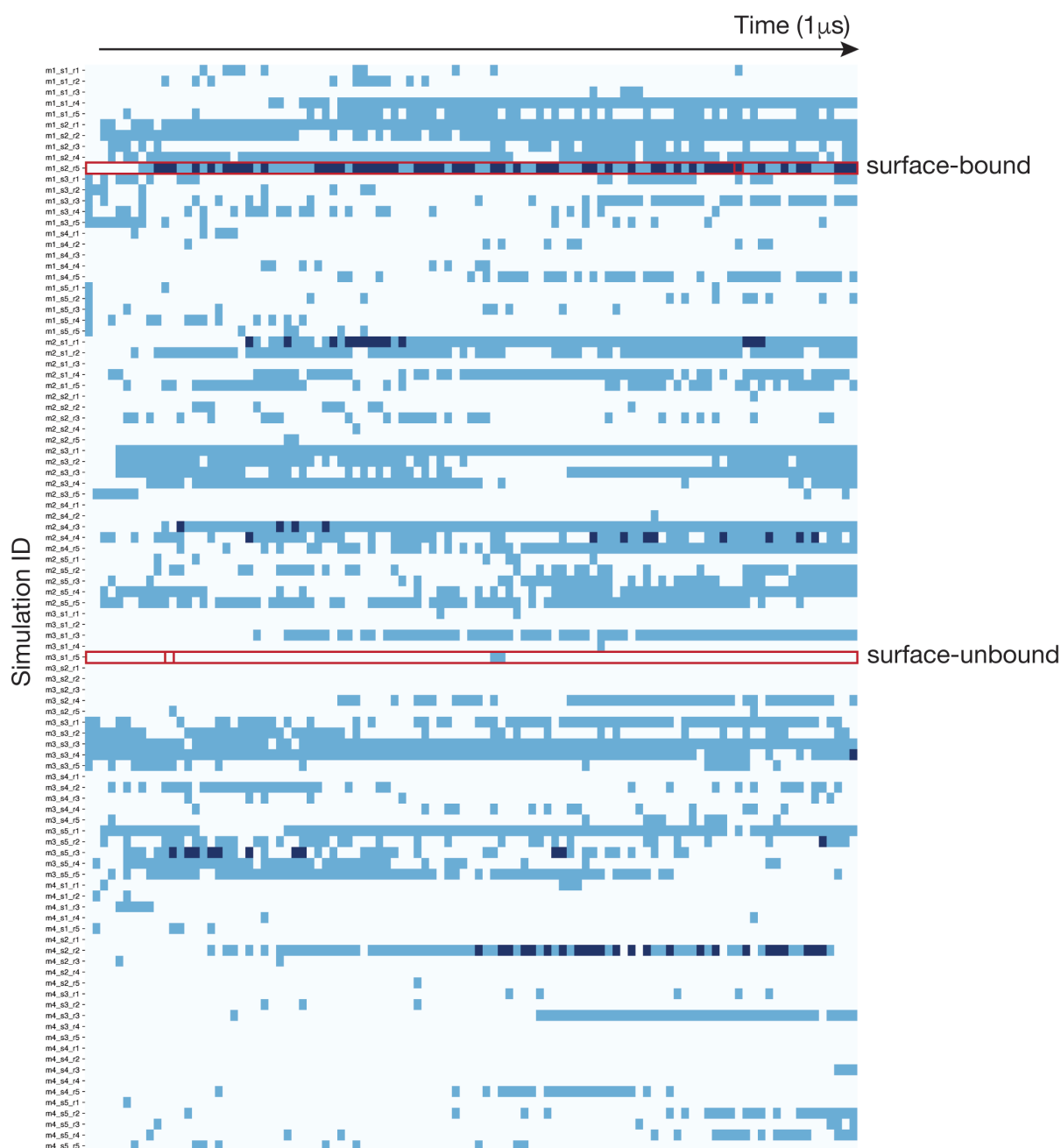


Figure S3. Heatmap representation of the cavity opening events. Snapshots, during the 100 μs of MD simulation, in which Phe283^{7.37} adopts the *trans* conformation or/and the distance between the top of TMs 1 (C α atom of Thr34^{1.33}) and 7 (C α atom of Val277^{7.31}) increases from the initial value of 14.5 Å to values larger than 15.5 Å. Light and dark blue indicates whether one or both events are happening, respectively. The trajectory in which the ligand (orange in Figure 1b) spontaneously binds the lipid-facing part of TMs 1 and 7 is highlighted as surface-bound, whereas a trajectory in which these events rarely occur is highlighted as surface-unbound. The selected snapshots for closed and open cavity in Figure 1e are highlighted by a red rectangle.

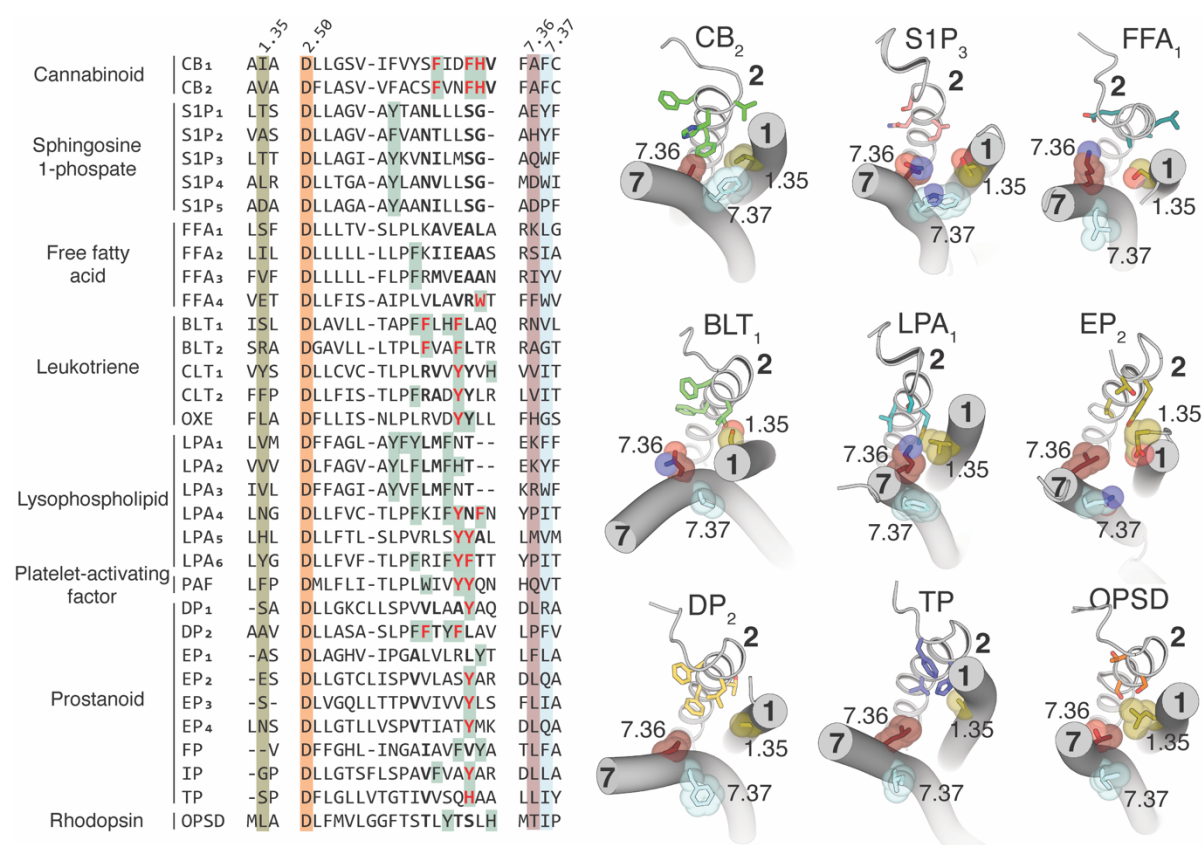


Figure S4. Structure-based sequence alignment of the amino acids forming the pathway of ligand entry to CB₂R among the members of class A GPCRs that bind hormone-like signaling molecules derived from lipid species. Sequence alignment of 3 positions of TM 1, 18 positions of TM 2, and 4 positions of TM 7 of the different lipid receptors, grouped by subfamily (left panel). The amino acids involve in the tunnel entrance (1.36 and 7.36) are shown, the amino acids closing/opening the tunnel (7.37) is shown, and aromatic residues in top 10 residues in TM 2 are shown. Residues in TM 2 pointing towards TMs 1 and 7 are highlighted in bold; aromatic residues within this selection are colored in red. Spatial visualization of these residues in different structures (right panels).